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Single-Cell RNA Sequencing of Mutant Whole Mouse Embryos: From the Epiblast to the End of Gastrulation

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Abstract

Over the last decade, single-cell approaches have become the gold standard for studying gene expression dynamics, cell heterogeneity, and cell states within samples. Before single-cell advances, the feasibility of capturing the dynamic cellular landscape and rapid cell transitions during early development was limited. In this paper, a robust pipeline was designed to perform single-cell and nuclei analysis on mouse embryos from embryonic day E6.5 to E8, corresponding to the onset and completion of gastrulation. Gastrulation is a fundamental process during development that establishes the three germinal layers: mesoderm, ectoderm, and endoderm, which are essential for organogenesis. Extensive literature is available on single-cell omics applied to wild-type perigastrulating embryos. However, single-cell analysis of mutant embryos is still scarce and often limited to FACS-sorted populations. This is partially due to the technical constraints associated with the need for genotyping, timed pregnancies, the count of embryos with desired genotypes per pregnancy, and the number of cells per embryo at these stages. Here, a methodology is presented designed to overcome these limitations. This method establishes breeding and timed pregnancy guidelines to achieve a higher chance of synchronized pregnancies with desired genotypes. Optimization steps in the embryo isolation process coupled with a same-day genotyping protocol (3 h) allow for microdroplet-based single-cell to be performed on the same day, ensuring the high viability of cells and robust results. This method further includes guidelines for optimal nuclei isolations from embryos. Thus, these approaches increase the feasibility of single-cell approaches of mutant embryos at the gastrulation stage. We anticipate that this method will facilitate the analysis of how mutations shape the cellular landscape of the gastrula.

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Disclosures

The authors have nothing to disclose.

Introduction

Gastrulation is a fundamental process required for normal development. This rapid and dynamic process occurs when pluripotent cells transition into lineage-specific precursors that define how organs form. For years, gastrulation was long defined as the formation of three largely homogeneous populations: mesoderm, ectoderm, and endoderm. However, high-resolution technologies and an emerging number of embryonic stem cell models[1,2] unveil unprecedented heterogeneity among the early germ layers[3,4]. This suggests that much more remains to be uncovered about the mechanisms regulating the distinct cell populations of the gastrula. Mouse embryonic development has been one of the best models to study early cell fate decisions during gastrulation[3,5]. Gastrulation in mice is rapid, as the entire process of gastrulation occurs within 48 h, from embryonic day E6.5 to E8[5].

Recent advancements in single-cell technologies have enabled detailed mapping of wild-type mouse embryonic development, providing a comprehensive overview of the cellular and molecular landscapes of embryos during gastrulation[3,4,6,7,8]. However, the analysis of mutant embryos at these stages is less common and often limited to FACS-sorted populations[9,10]. The scarce literature reflects the technical challenges associated with the manipulation and single-cell preparation of gastrulating embryos that require genotyping. Capturing the dynamic process of gastrulation can pose challenges due to its rapid nature, especially for understanding mutant embryos. The timing and synchronization of pregnancies are essential, as even slight differences between timed pregnancies can be misinterpreted as a developmental phenotype resulting from the mutant gene. This becomes particularly important when the mutant gene influences the process of gastrulation[13,14]. In this study, guidelines are established to obtain synchronized pregnancies through visualization of vaginal plugs (i.e., the mass of coagulated seminal fluid formed in the female's vagina after mating). Additionally, a strategy is designed to obtain robust single-cell data from mutant gastrulating embryos from E6.5 to E8. This strategy is devised to overcome constraints associated with the low number of embryos with the desired genotype per pregnancy and the decrease in viability caused by freezing-thawing embryos or cells.

This paper describes an optimized methodology from the establishment of timed pregnancies via vaginal plugs to the final sequencing of single cells/nuclei. This method explains how to increase the number of synchronized pregnancies to obtain a higher number of embryos with desired genotype, cell/nuclei isolations to improve the viability of the cells, and a same-day genotyping protocol. This manuscript also describes the process of embryo isolation at different gastrulation time points. The methodology helps to increase the number of final viable embryo cells/nuclei for sequencing, ensuring high-quality sequencing data. Therefore, this method will open the doors for single-cell studies of gastrulating embryos that require genotyping.

Protocol

This protocol and all animal experiments described were formally approved and in accordance with institutional guidelines established by the Temple University Institutional Animal Care and Use Committee, which follows the Association for Assessment and

Accreditation of Laboratory Animal Care international guidelines. All mice described were on the C57/BL6N background strain. No animal health concerns were observed in these studies.

1. Breeding colony and timed pregnancies

1. Time the pregnancies by the visualization of a vaginal plug. Noon on the day of the plug is considered E0.5.
2. House mice in cages with bedding material containing chipped hardwood bedding and paper nesting material.
    1. Each cage contains mouse chow, fresh water, and an enrichment item (e.g., tunnel and nesting material). Track and collect colony information daily during timed breeding.
    2. Log all information such as the age of the mouse, the number of pregnancies a female mouse has had, the number of plugs placed by a male mouse, and the stage of estrous (Figure 2) for female mice.

        NOTE: Females that have already given birth 1-2 times will deliver larger litter sizes in future pregnancies.
3. Before starting timed pregnancies, house the male mouse alone in a cage 1 week prior to breeding. During the week of breeding initiation, introduce at least 1 female per male in the cage.

    NOTE: Depending on the approved animal protocol, adding 2-3 females into a breeding cage may be allowed and is preferred to increase plug generation.
4. Set up at least 4 breeding trios (2 female mice to 1 male) to increase the chances of synchronized pregnancies across the cages (aka., multiple plugs in the same day). This will increase the number of embryos isolated at the same developmental stage.
5. Arrange ideal mating in the afternoon or evening before 5 PM, and females are placed into the male's cage or vice versa. If breeding does not occur in 4-5 days, consider switching mating partners every other day.

    NOTE: If unable to check for a vaginal plug the following morning, separate the breeding pair the night before (i.e., weekend/holidays).
6. Check for vaginal plugs every day in the early morning; before 9 AM is preferred.
    1. To check for vaginal plugs, gently lift the female mice by the base of the tail and observe the vaginal opening. Look for a white or cream-colored gelatinous mass. Often, the vaginal plug can be obvious, but if unclear, take tweezers and gently probe the vaginal opening. Consider only the more apparent plugs for isolation. Refer to Figure 2C.

NOTE: It is critical to check for vaginal plugs as early as possible in the morning to avoid missing a potential pregnancy. Plugs can fall out or dissolve after 12 h.

NOTE: Even if a plug is observed, it does not guarantee that the female mouse will be pregnant. If a partial or no plug is observed but there is redness near the vaginal opening of the female mice, do not consider these females for isolation as there is a less likely chance the plug will stick. The likelihood of pregnancy after mating varies among mouse strains and depends on the phase of the estrous cycle during mating (Figure 2).

7. Once a vaginal plug is observed, record the day. The noon of the same day that the vaginal plug is observed is considered E0.5. Separate the female mice from the breeding cage and isolate the embryos depending on the stage of gastrulation desired.

   NOTE: This method does not provide precise timing for mating. Conventionally, mating is assumed to occur around midnight the preceding night. Consequently, embryos are considered to be half a day old (E0.5) by noon on the day when the vaginal plug is observed.

2. Isolation of mouse embryos during gastrulation

1. Prior to starting the embryo isolation, prepare all required reagents and equipment.
   1. Clean the area thoroughly with 70% ethanol. Ensure all dissection tools (forceps and scissors) are washed and sterilized. Obtain sterile 5 mL of Dulbecco's Modified Eagle Medium (DMEM)/10% fetal bovine serum (FBS) and 20 mL $DPBS^{-/-}$ and place on ice.
   2. Perform all isolations using a stereomicroscope with a transmitted light stage and camera to assist with gross developmental phenotyping.
2. Euthanize the pregnant mouse dam and start the isolation immediately.
   1. Place the pregnant dam in a CO_2 chamber with the CO_2 flow rate adjusted to displace 20% of the cage volume per minute. Monitor the mouse closely and confirm death by observing the absence of breathing movements.
   2. Maintain CO_2 flow for an additional 2 min after the absence of breathing movements. Confirm euthanasia by performing a cervical dislocation.
   3. Position the mice in a normal standing position on a firm, flat surface. Then, with the thumb and first finger of one hand against the back of the neck at the base of the skull, push forward and downward while pulling backward with the other hand holding the tail base.

4. Verify the effectiveness of dislocation by feeling the separation of cervical vertebrae. When the spinal cord is severed, a 2-4 mm space will be palpable between the occipital condyles and the first cervical vertebra.

   NOTE: Do not euthanize multiple pregnant dams at once, as cell viability will be affected. Isofluorane can be used as an alternative anesthetic agent if approved by the Institutional Animal Care and Use Committee (IACUC) or equivalent body.

3. Place the pregnant dam on its back and sterilize the area near the vaginal opening with ethanol.

   1. Using dissection scissors and tweezers, lift the skin fold near the vaginal opening and make a small V-shaped cut, slowly revealing the uterus of the pregnant dam (Figure 3 [yellow arrow]).

   2. Dissect out the uterine horn of the pregnant dam by holding one end of it with tweezers and cutting along it, making sure to remove the cervix. Place the uterine horn into a 10 cm Petri dish containing $DPBS^{-/-}$ on ice (Figure 3).

      NOTE: Depending on the embryo isolation stage, the uterus will resemble smaller or larger implantation sites (i.e., 'pearls' on a string).

4. With dissection scissors and tweezers, cut each implantation site ('pearls') containing the decidual swellings inside and place into fresh $DPBS^{-/-}$ in a 6 cm Petri dish on ice (Figure 3).

   NOTE: An average pregnant dam will have around 6-8 implanted embryos.

5. Take one implantation site and place it on a new 6 cm Petri dish on top of the stereomicroscope stage and add 500 µL of $DPBS^{-/-}$ on top of it. Adjust the focus of the microscope and the light source (Figure 3).

   NOTE: Depending on the size of the implantation site, the amount of DPBS might vary; the goal is to have enough DPBS that the implantation site is submerged.

6. Using fine-tipped dissection tweezers, remove the uterine muscle from the implantation site. Hold down the implantation site with one set of tweezers in one hand and slowly insert another pair of forceps with the other hand into the end of the implantation site cut from the uterine horn, slowly revealing the decidua swelling (Figure 3).

   NOTE: Do not pull or tug too hard on the uterine surface, as it can lead to the rupture of the decidual swelling or even the lysis of the embryo.

7. After the decidual swelling is isolated, proceed to reveal the embryo.

   1. Hold the anti-mesometrial end of the decidual swelling with one pair of forceps and, with the other pair, slowly make a horizontal cut about ¼

the size of the decidual swelling from the mesometrial end (i.e., often the more pointed end of the decidual swelling).

2. Now, with both forceps, slowly push from the anti-mesometrial end of the decidual swelling, and the embryo will pop out from the freshly cut mesometrial end (Figure 3).

   NOTE: Do not tear into the decidual swelling, as this will break the embryo. If necessary, make smaller cuts along the mesometrial end of the decidual swelling.

8. Once the embryo is revealed, remove any extraembryonic tissues attached.

   1. The parietal endodermal sac and ectoplacental cone might spontaneously come off from the embryo during the revealing process, but if not, use a pair of forceps and remove them along with any associated maternal blood. Then, using two forceps, hold down the embryo with one pair and slowly peel the visceral yolk sac from the embryo using the other set.

   2. Using a P20 pipette, place the yolk sac with no more than 10 µL of $DPBS^{-/-}$ from the dish into an 8-strip polymerase chain reaction (PCR) tube on ice, as this will be used for same-day genotyping.

      NOTE: It is critical that the yolk sac sample is not contaminated with tissue from the pregnant dam. Contamination may lead to incorrect genotype assignment of the embryo.

9. Take bright field pictures of freshly isolated embryos to ensure the staging of the littermates is similar. With a P200 pipette, slowly pipette up the embryo with 50 µL of DMEM/10% FBS and place it into a 1.5 mL tube on ice. Let the embryos remain on ice until genotypes have been confirmed.

   NOTE: Embryos were kept on ice for 3-4 h with no obvious degradation, but do not exceed this time, as a decrease in cell viability will occur. Label the embryos and genotyping tubes accordingly. Taking bright field images is encouraged to identify and annotate gross phenotypic differences among embryos.

10. Repeat these steps for all remaining decidual swellings. Clean all dissection tools and use new plastics for every isolation to ensure no contamination from previous isolations.

    NOTE: Ensure that the dissection procedures are limited to 1 h from the moment of collection of the implantation site from the pregnant dam.

11. Proceed to isolate embryos from the next pregnant dam (if multiple synchronized pregnancies were identified) before moving to the same-day genotyping step.

3. Same-day genotyping (Figure 4)

1. Digest each visceral yolk sac in an 8-strip PCR tube. Using a P20 pipette, add 19.3 µL of PCR template DNA lysis buffer and 0.7 µL of 0.2 µg/mL Proteinase K to each yolk sac sample.

2. Vortex the sample for 10 s and spin down to position the samples at the bottom of the tube using a mini centrifuge with a strip adaptor at 1,000 x *g* for 10 s. Place the 8-strip PCR tube in an 85 °C heat block for 45 min and vortex for 5 s every 5 min.

   NOTE: It is important to vortex samples while digesting to maximize cell lysis in 45 min.

3. After 45 min, spin the tube strip down using a mini centrifuge with a strip adaptor at 1,000 x *g* for 10 s, and proceed with PCR for desired genetic identification. For reference, a sample protocol for a Cre-lox system is provided. The following is an example of PCR conditions for Cre genotyping. Design primers to amplify the 5' and 3' regions of the targeted Cre site.

4. To perform the PCR reaction for Cre genotyping, prepare a PCR master mix per 8-strip PCR tube for each yolk sac containing 10 µL of Taq DNA polymerase mix, 0.5 µL of 5 µM forward Cre primer, 0.5 µL of 0.5 µM reverse Cre primer, 5 µL of PCR-certified water, and 4 µL of yolk sac genomic DNA.

   NOTE: If using a protocol that was optimized for mouse tail genotyping, add double the amount of DNA that is typically utilized for cleaner results.

5. Once the PCR master mix has been made, run the PCR thermal cycle amplification program. For Cre genotyping, the cycle is as follows: (1) 95 °C for 3 min, (2) 95 °C for 30 s, (3) 55 °C for 30 s, (4) 72 °C for 30 s, repeated step 2-4 for 34 cycles, (5) 72 °C for 10 min, (6) 4 °C hold. Run the PCR products on a 1% agarose gel to draw genotyping conclusions.

   NOTE: If more than one PCR is necessary for genetic identification, run the PCR reactions simultaneously to optimize timing. To expedite the process, prepare the agarose gel a day in advance on the day of the experiment and store it at 4 °C overnight. Do not consider any samples without clear genotypes. Take both the genotype and developmental stage into consideration for samples, as littermates could be at different stages of gastrulation and potentially skew results. When performing the genotyping of the LoxP alleles, the PCR bands expected in the gel may vary depending on the Cre-driver used. For instance, if the Cre driver is expressed in the visceral yolk sac, the Loxp band will appear shifted in the Cre-positive (Cre+) embryos, compared to the Cre-negative (Cre−). However, if the Cre is not expressed in the visceral yolk sac, the size of the Loxp band will be the same size in the Cre+ and the Cre− embryos (i.e., the Loxp alleles will not be floxed in the yolk sac lineage). An embryo carrying one Cre+ allele and two Loxp alleles is considered a conditional KO embryo. However, to confirm the deletion of the floxed gene, it is recommended to perform a confirmation of the knockout of the gene on the cells that express the Cre driver, either by repeating

the genotyping protocol or by qPCR analysis of the mRNA levels of the floxed gene (Figure 4D, E).

6. Store the remaining digested yolk sac samples in the −20 °C freezer for long-term storage.

4. Cell dissociation of embryos and cell viability

1. Once the genotypes have been confirmed, take a P200 pipette and pipette 50 µL of DMEM/10% FBS. Pool embryos with the same genotype into a new 1.5 mL tube and place the tube on ice.

   NOTE: Do not proceed with the experiment if there are not at least 5 embryos per group (E7-E7.5) or 3 (E7.75-E8), as cell count and viability will decrease tremendously.

2. After embryos have been pooled based on genotypes, allow them to settle to the bottom of the tube. Wash the pooled embryos by adding 50 µL of $DPBS^{-/-}$, then wait for the embryos to settle before removing as much of $DPBS^{-/-}$ as possible without removing the embryos from the tube. Repeat this step twice.

   NOTE: Holding the 1.5 mL tube up to a light source or towards a window will make it easier to see the embryos settling to the bottom of the tube.

3. Add 100 µL of trypsin to the pooled embryos and incubate at 37 °C in a heat block for 5 min. Gently flick the 1.5 mL tubes every 30 s to help the cells dissociate.

   NOTE: Do not use a vortex or a pipette during the trypsinization process, as it damages the cells. If more than 5 embryos (E7-E7.5) or 3 embryos (E7.75-E8) are pooled, perform trypsin digestion in another tube with an equivalent amount of trypsin (~20 µL per 1 embryo). Use ~20 µL of trypsin per embryo (E6.5-E7.5), 35 µL per embryo (E7.75), and 40 µL for embryo (E8).

4. After 5 min, neutralize the trypsin with 300 µL of DMEM/10% FBS. Centrifuge the pooled embryos at 100 x *g* for 4 min at room temperature (RT). After centrifugation, a small pellet will appear for all samples. Resuspend the pellets in 40 µL of DMEM/10% FBS and place them on ice.

   NOTE: The size of the pellet will vary depending on the number of embryos pooled. It is possible that the pellet is not visible but proceed with the next step.The amount of DMEM/10% FBS required to neutralize trypsin will depend on the amount of trypsin added. Add 3 times the amount of DMEM/10% to the trypsin amount.

5. Determine the concentration of the resuspended cells (40 µL) using an automated cell counter. Mix 5 µL of cells with 5 µL of trypan blue in a new 1.5 mL tube. Pipette mix thoroughly and pipette onto a slide to determine the cell number and cell viability. The optimal concentration of cells is 700-1200 cells/µL, and the cell viability is 90% or higher.

NOTE: If the concentration is lower than 200 cells/µL and viability is lower than 50%, do not continue the experiment. If cell concentration is too high, dilute the cell suspension and recount cells again. If cell clumps are observed, use a cell strainer to ensure a single-cell suspension.

6. Proceed to single-cell partitioning using a microfluidic chip and follow the protocol from the microfluidic chip manufacturers[11].

5. Nuclei isolation mouse embryos (option for larger embryonic time points from E8 onward)

1. Prepare fresh lysis and wash buffers outlined in Table 1, and place them on ice.

   NOTE: Nuclei isolation can be performed on fresh or frozen samples. If samples are frozen, allow them to thaw for 2-5 min on ice.

2. Prior to starting the experiment, confirm all genotypes and pool only embryos with clear genotypes.

3. Using a P200 pipette, add 50 µL of nuclei lysis buffer to pooled embryos in a 1.5 mL tube, aiming for a minimum of 3 embryos per mutant group. Allow samples to sit on ice with lysis buffer for 5 min and vortex every 30 s.

4. After 5 min of incubation, centrifuge at 500 x *g* for 5 min at 4 °C. Using a P200 pipette, remove the supernatant and resuspend the pellet in 50 µL of wash buffer. Once resuspended, centrifuge at 500 x *g* for 5 min at 4 °C.

5. Remove the supernatant and resuspend in 40 µL of $DPBS^{-/-}$. Count the cells using an automated cell counter. Mix 5 µL of nuclei with 5 µL of trypan blue in a new 1.5 mL tube. Pipette the mixture thoroughly and load it onto a slide to determine the cell number and viability.

   NOTE: The nuclear membrane is permeable to trypan blue; therefore, the isolated nuclei stain is positive for trypan blue. In an automated cell counter, the percentage of dead cells is used to estimate the percentage of isolated nuclei (Figure 5A).

6. If the percentage of intact nuclei is greater than 90% (meaning that at least 90% of the nuclei are stained with trypan blue and exhibit a rounded shape and turgid aspect; refer to Figure 5B, C), proceed to utilize a microfluidic chip and follow protocol from the microfluidic chip manufacturers[11].

6. Single-cell partitioning (including cDNA amplification and library construction)

1. Proceed immediately with the single-cell RNA sequencing protocol following the microfluidic chip manufacturer's protocol[11] for the most optimal results. Set the target cell recovery to 6000 cells or greater.

7. Sequencing

1. After libraries are constructed, measure the fragment size distribution and concentrations of samples using an automated electrophoresis analyzer.

NOTE: Optimal fragment size distributions are between 300-1000 bp.

2. Samples are ready to be loaded into the sequencer. Pool libraries from different samples together. The final loading concentration of pooled libraries is 750 pM in a total volume of 24 µL. Load the pooled libraries into the reagent cartridge, following the sequencing manufacturer's protocol[12].

3. Sequence the libraries loaded into the pre-assembled flow cell and cartridge according to the desired sequencing depth with pair-end, dual indexing.
The sequencing reads adhered to the protocol outlined by microfluidic chip manufacturers is as follows[11]: Read 1: 28 cycles, i7 Index: 10 cycles, i5 Index: 10 cycles, and Read 2: 90 cycles. Following the completion of sequencing, the data undergoes bioinformatics analysis.

4. Use bioinformatics methods to perform quality controls. This includes assessing the number of sequenced cells, reads per cell, and number of genes mapped per cell.

   NOTE: For optimal results, the number of sequenced cells is at least 80% of the targeted cells. It is recommended that the number of reads per cell is at least 30,000, and the number of genes detected higher than 3000 (for mouse samples).

Representative Results

The methodology designed in this paper is specifically intended to enhance the preparation of embryo samples for single-cell omics from E6.5 to E8. This robust pipeline consists of five major steps: synchronized timed pregnancies, embryo isolations, same-day genotyping, cell dissociation, and assessment of cell viability (Figure 1A). While the presented data focuses on time points from E7 to E7.5, it can be applied to embryos up to E8 (Figure 1B) with small variations in the procedure (referred to notes throughout the protocol). Synchronized timed pregnancies were achieved by placing two female mice into a cage with a male mouse. Vaginal plugs were checked every morning before 10 AM, and if a plug was observed, it was considered E0.5 by noon of that day (Figure 2A). In this study, optimal conditions for plugs required a female mouse in the estrus or proestrus stage to be paired with a male with a history of successfully placing several plugs during prior breeding (Figure 2B). Only obvious plugs were considered for embryo isolations (Figure 2C).

The developmental timing was estimated following the timetable in Figure 2A. For E7.5, embryo dissection started at 12 PM on the 7th day following the day of the detection of a plug. Figure 3 exemplifies a successful embryo isolation at E7.5. After the pregnant dam was euthanized, the uterine horn was dissected, the decidual swelling was individually cut, revealing the embryo, and the yolk sack was isolated for genotyping (Figure 3).

Same-day genotyping was performed within 3 h of embryo isolation following the steps in Figure 4A. The embryos were kept on ice during the process of genotyping to preserve their integrity. Do not freeze the embryos, as it will decrease the number of viable cells per embryo. Figure 4B,C show the visceral yolk sack, the parietal endodermal sac, and the ectoplacental cone with associated maternal blood. The visceral yolk sac was utilized for genotyping (Figure 4C). After digesting the yolk sac, the PCR mix was prepared, and the

PCR reaction was run. The resulting PCR product was then separated on an agarose gel. Figure 4D shows expected fragment sizes for the LoxP and wild-type alleles (597 bp and 498 bp, respectively) and the Cre allele (650 bp). In Figure 4E, an example of yolk sac genotyping from 6 embryos obtained from breeding pairs carrying Cre and LoxP (flox) alleles is presented. The gels depict the PCR products of the Cre and LoxP alleles in the 6 embryos analyzed. Embryos number 2 and number 5 carry 2 flox alleles and 1 cre allele; therefore, they are considered conditional KO embryos (Figure 4E, red dots). Embryos 1 and 3 have 2 flox alleles but are negative for Cre; therefore, they are considered flox controls. Embryo 4 has 2 flox alleles and a faint band for the Cre allele, resulting in an "unclear" genotype. This embryo was not further processed (alternatively, the experimentalist may consider repeating the genotyping or conducting a secondary validation of the conditional KO using cells expressing Cre). It is important to note that the Cre driver used in this experiment is not expressed in the visceral yolk sac[15]; hence, the LoxP alleles do not appear shifted on the gel of Cre-positive embryos compared to the Cre-negative ones.

Cell count and good viability are required for a successful single-cell experiment. Suspensions with low cell viability, a high percentage of dead cells, clumping, or significant debris are unsuitable for further processing. Optimal conditions are for 700-1200 live cells per microliter and >90% viability. Figure 5A presents a panel illustrating both good and sub-optimal cell viability. The same criteria can also be applied for nuclei isolation, as shown in Figure 5B. However, it is crucial to note that the evaluation of trypan blue differs from cells and nuclei: viable cells do not incorporate trypan blue, while viable nuclei do. If cells/nuclei suspension is optimal (>90% viability), proceed with single-cell partitioning using a microfluidic chip following manufacturer's procedures[11]. Troubleshooting options are provided for suspensions where cell/nuclei viability is between 60%-89%, as depicted in Figure 5C. If viability, regardless of the total number of cells, falls below 60%, consider halting the experiment.

The entire pipeline from the culling of the pregnant dam to library construction takes a total of 8 hours on the same day (i.e., protocol steps 2 to 6 must be performed on the same day). Following the procedures outlined by the single-cell manufacturers' procedures[11], library constructions were prepared for single-cell RNA sequencing using cells obtained from E7 embryos, with cell viability ranging from sub-optimal to optimal conditions, aiming for an estimated target recovery of 2000 cells (Figure 6). Figure 6A depicts a representative fragment size distribution of scRNAseq libraries for both sub-optimal and optimal conditions, indicating that cell viability does not significantly affect the entire single-cell partitioning process. The fragment size distribution ranged between 400-500 bp. This indicates that sub-optimal conditions do not affect the process of library preparation. Figure 6B shows the outcomes of both successful and sub-optimal single-cell RNAseq experiments. Following sequencing, quality control checks were conducted on samples and observed that, in cases where cell viability was sub-optimal, only 10% of cells were successfully sequenced. In contrast, optimal samples exhibited a higher percentage, with 91% of the total cells being sequenced. This is further proven by barcode plots, indicating that the sub-optimal conditions have larger background noise compared to optimal conditions. Clustering analysis was performed for both experiments and revealed 9 clusters in the optimal conditions and 4 in the sub-optimal. Annotation of the clusters using known

markers[3] revealed expected cell -types in the E7 embryos, including epiblast and primitive streak (Figure 6B). Cluster annotations in the suboptimal experiment were not possible due to the lack of enrichment in known markers for each cluster. This highlights the importance of high-quality cells required for the proper representation of data during these stages of development.

Discussion

A robust pipeline is presented in this paper for obtaining high-quality single-cell and nuclei suspensions from gastrulating mouse embryos, specifically designed to facilitate studies on mechanisms of cell-fate specification in early development. This method addresses a crucial gap in the field of gastrulation by optimizing the analysis of embryos requiring genotypes, such as sex or somatic genes. By utilizing genetic mutation mouse models and employing high-resolution single-cell sequencing on whole mouse embryos, this pipeline can further enhance the understanding of the gene expression profiles of the early mouse gastrula. This method demonstrates the feasibility of using a genetic mutation mouse model by a Cre recombinase system to obtain high-quality cells and nuclei at early developmental time points for single-cell omics. This method evolved through multiple attempts, during which samples did not meet the quality standards required for library preparation and sequencing. The explained methodology generates sufficient cells/nuclei from embryos younger than E8 through the optimization of three critical steps: (1) synchronized timed pregnancies to increase the number of embryos, (2) same-day genotyping to avoid freezing/thawing, and (3) assessing cell/nuclei viability to avoid sequencing of dying cells. This protocol delivers successful results with single-cell RNASeq, but the cell or nuclei suspensions obtained in this protocol can be processed in other sequencing platforms, where cell viability is the limiting factor, such as smart-seq, drop-seq, and cel-seq[16].

At the E7 embryo, the number of cells can vary depending on the strain and mutant genotype. Typically, a E7 embryo can range from hundreds to a few thousand cells, and obtaining embryos with the desired genotype is challenging, with only 1 or 2 embryos per pregnant dam meeting the criteria. This protocol allows the capture of around 300 viable cells per E7 embryo for single-cell analysis. Attempts to increase embryo pool size by snap-freezing embryos from pregnant dams on different days proved unsuccessful, as cell viability was severely compromised after thawing, even with the addition of cryopreserving agents. To address this challenge, the breeding strategy and synchronization of pregnant dams was optimized. To increase the chances of multiple isolations, it is recommended to use female mice that have given birth 1-2 times before breeding for isolation, as they are more likely to have larger litter sizes. Monitoring the female's estrous cycle is crucial; mating is more likely to occur during the proestrus and estrus stages. If breeding difficulties arise, switching the breeding partners after four days will also help if no plug is produced.

It's important to note that this method has a limitation: it relies on observed plugs, and even if a plug is observed, it does not guarantee pregnancy but only indicates sexual activity. Therefore, increasing the number of observed plugs in a day will increase the probability of having more than one pregnant dam in a day and more positive genotypes. This protocol demonstrated the feasibility of using embryonic stages ranging from E7 to E7.75 as a proof

of concept during gastrulation. However, this pipeline can be applied from E6.5 to E8 embryos. For E6.5 embryos, it is recommended to increase the number of synchronized pregnancies to obtain at least 7 embryos with the desired genotype to pool. Instead, for the E8 embryos, increase the amount of trypsin used to dissociate each embryo to ~ 40 µL instead of 20 µL. The goal is to obtain a cell/nuclei suspension in a concentration range of 700-1200 cells per µl before proceeding with the partitioning.

Having good cell viability is essential for the success of single-cell sequencing. In the microfluidic chip design provided by the manufacturers, single cells are partitioned into gel beads-in-emulsion (GEMs) within a chip containing known barcoded gel beads[11]. However, a notable limitation of this process is that both high-quality and poor-quality single cells can be partitioned. Even with an adequate number of live cells (i.e., 1000 cells/µL), if the viability of the suspension is low (i.e., 1000 cells alive and 1000 cells dead, resulting in 50% viability), the sequencing experiment will likely fail. For optimal results, it is recommended to aim for a viability of around 90%. If the cell viability falls between around 60%-89%, specific measures can be taken to enhance the experiment's viability. However, if the cell viability is less than 60%, it is strongly advised against continuing with the experiment. The reason for this is that the dying cells will be 'captured' in the partitioning gel, and subsequent library preparation and quality controls will pass without noticeable issues. However, the actual sequencing experiment may completely fail, as most of the sequencing reads will map mitochondrial, ribosomal, or apoptosis genes, indicating signs of poor cell viability from the outset. Data presented in this paper illustrates a pipeline for single-cell sequencing of gastrulating mouse embryos with high-quality cells. This methodology can be applied for the understanding of many different genetic mutation mouse models during development.

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References

1. Arias AM, Marikawa Y, Moris N Gastruloids: Pluripotent stem cell models of mammalian gastrulation and embryo engineering. Dev Biol. 488, 35–46 (2022). [PubMed: 35537519]
2. Kim Y, Kim I, Shin K A new era of stem cell and developmental biology: from blastoids to synthetic embryos and beyond. Exp Mol Med. 55 (10), 2127–2137 (2023). [PubMed: 37779144]
3. Pijuan-Sala B. et al. A single-cell molecular map of mouse gastrulation and early organogenesis. Nature. 566 (7745), 490–495 (2019). [PubMed: 30787436]
4. Mittnenzweig M. et al. A single-embryo, single-cell time-resolved model for mouse gastrulation. Cell. 184 (11), P2825–2842.E22 (2021).
5. Bardot ES, Hadjantonakis AK Mouse gastrulation: Coordination of tissue patterning, specification and diversification of cell fate. Mech Dev. 163, 103617 (2020). [PubMed: 32473204]
6. Argelaguet R. et al. Multi-omics profiling of mouse gastrulation at single-cell resolution. Nature. 576 (7787), 487–491 (2019). [PubMed: 31827285]
7. Chan MM et al. Molecular recording of mammalian embryogenesis. Nature. 570 (7759), 77–82 (2019). [PubMed: 31086336]

8. Mohammed H et al. Single-cell landscape of transcriptional heterogeneity and cell fate decisions during mouse early gastrulation. Cell Rep. 20 (5), 1215–1228 (2017). [PubMed: 28768204]
9. Lescroart F. et al. Defining the earliest step of cardiovascular lineage segregation by single-cell RNAseq. Science. 359 (6380), 1177–1181 (2018). [PubMed: 29371425]
10. Scialdone A. et al. Resolving early mesoderm diversification through single-cell expression profiling. Nature. 535 (7611), 289–293 (2016). [PubMed: 27383781]
11. 10X Genomics. Chromium Next GEM single cell 3' reagent kits v3.1: User guide. at <https://www.10xgenomics.com/support/single-cell-gene-expression/documentation/steps/library-prep/chromium-single-cell-3-reagent-kits-user-guide-v-3-1-chemistry> (2024).
12. Illumina. NextSeq 1000/2000 Sequencing v2.0 Protocol. at <https://support-docs.illumina.com/SW/ClarityLIMS-INT/Content/SW/ClarityLIMS/Integrations/NextSeq10002000Intv20Config.htm> (2024).
13. Dai HQ et al. TET-mediated DNA demethylation controls gastrulation by regulating Lefty-Nodal signalling. Nature. 538, 528–532 (2016). [PubMed: 27760115]
14. Li Q. et al. Base editing-mediated one-step inactivation of the *Dnmt* gene family reveals critical roles of DNA methylation during mouse gastrulation. Nat Commun. 14 (1), 2922 (2023). [PubMed: 37217538]
15. Saga Y. et al. MesP1 is expressed in the heart precursor cells and required for the formation of a single heart tube. Development. 126 (15), 3437–3447 (1999). [PubMed: 10393122]
16. Ziegenhain C. et al. Comparative analysis of single-cell RNA sequencing methods. Mol Cell. 65 (4), 631–643.e4 (2017). [PubMed: 28212749]

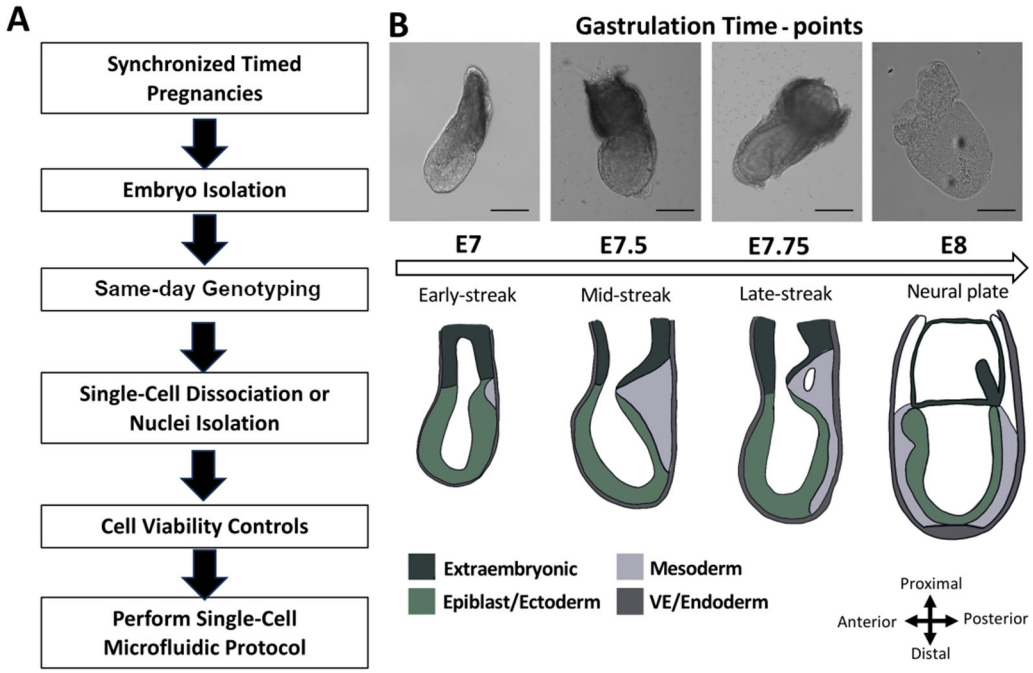

Figure 1: Optimization of gastrulating whole mouse embryos for single-cell RNA sequencing.

(**A**) Workflow schematic for obtaining high-quality cells and/or nuclei from gastrulating embryos. (**B**) Representative bright field images and adapted scheme[3] of mouse embryos during gastrulation from E7 to E8. Scale bar: 125 μm.

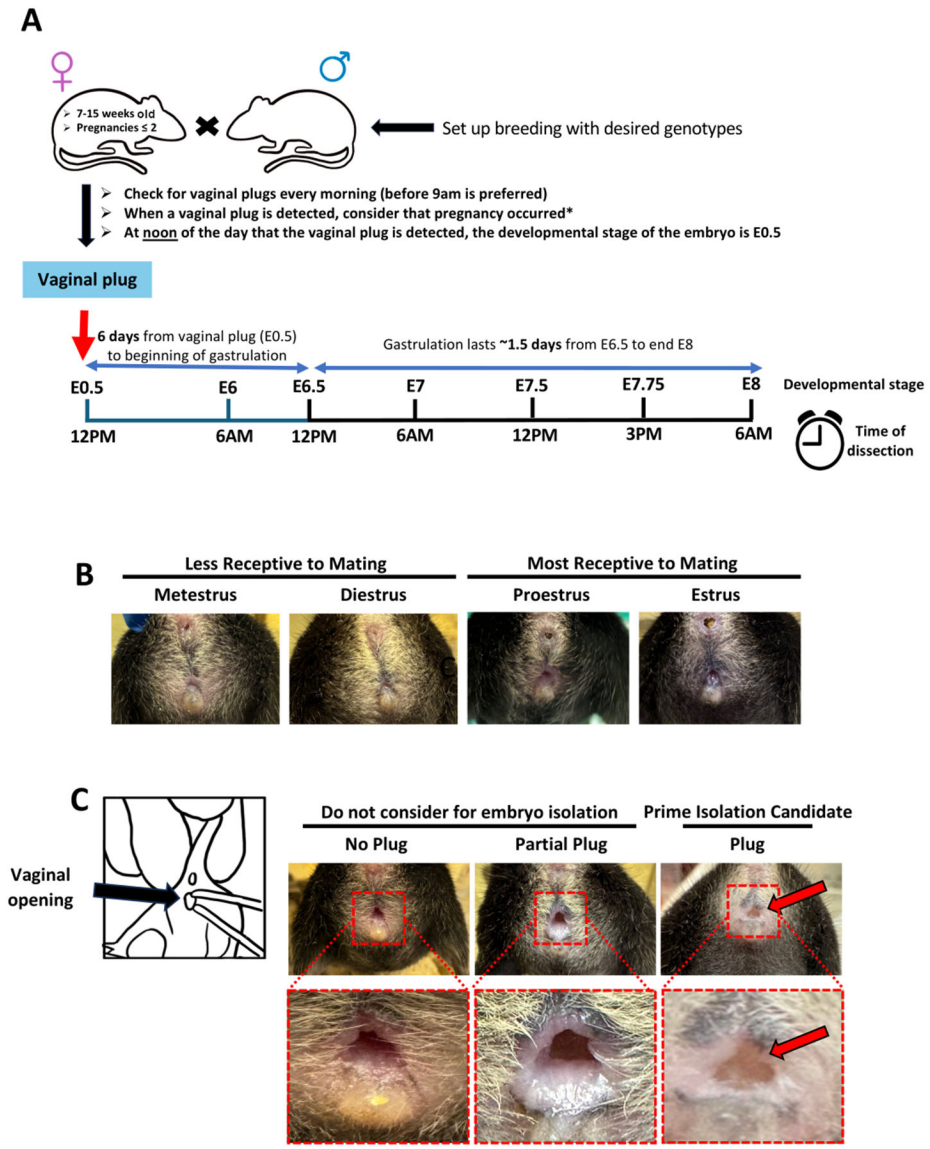

Figure 2: Strategy for efficient synchronized timed pregnancies for embryo isolations during gastrulation.

(**A**) Schematic diagram indicating the pipeline for timed pregnancies and a timetable describing the times from plug detection to embryo isolation for analysis of specific gastrulation phases. (**B**) Representative images of the phases of the estrus cycle for female mice. The phases most receptive to breeding are indicated. (**C**) Schematic diagram illustrating how to check for vaginal plugs and examples of vaginal openings without a plug, a partial plug, or a good plug to consider for embryo isolations. The vaginal openings are zoomed in below each image. The plug (coagulated semen in the vagina opening) is highlighted with a red arrow pointing to it.

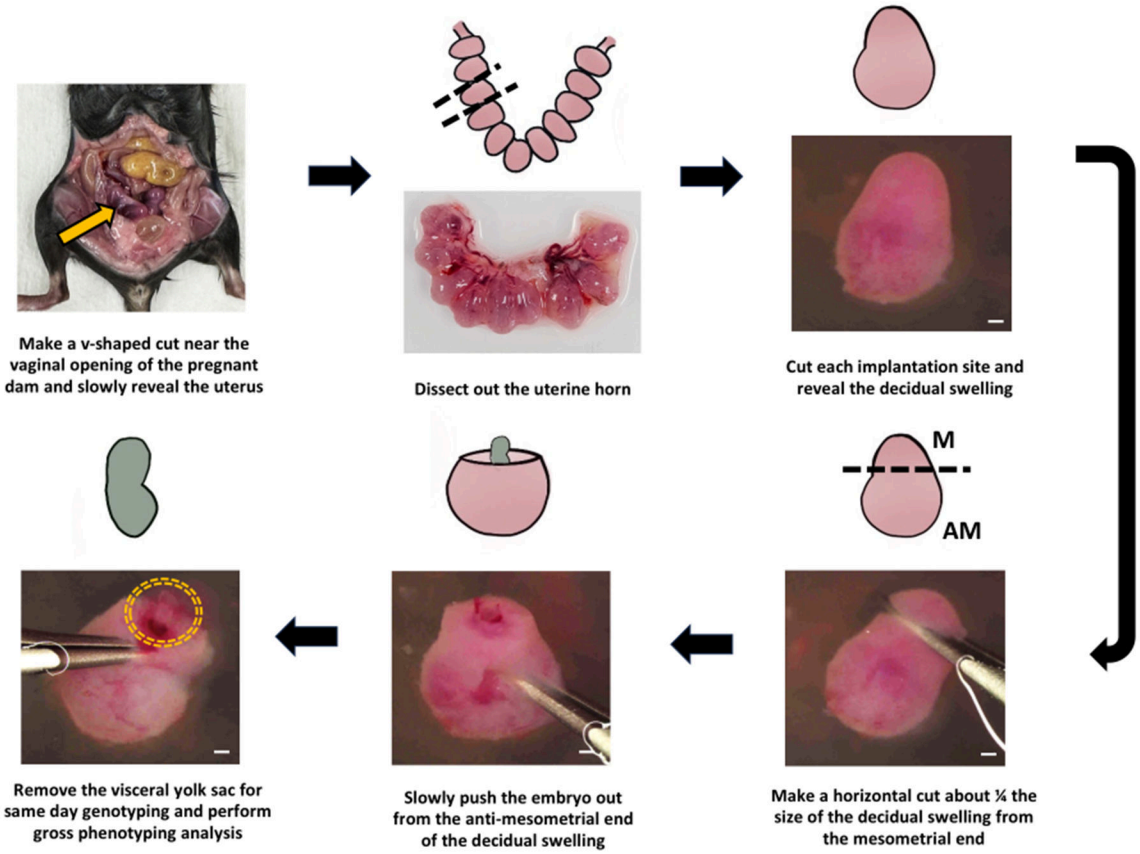

Figure 3: Dissection and genotyping of E7.5 embryos for single-cell RNA sequencing.

Schematic diagrams and images of the process of isolating E7.5 embryos and dissecting the visceral yolk sac. The yellow arrow indicates the uterus of the pregnant dam, and the yellow dashed circle outlines the location of the embryo. Dashed lines represent the areas that were cut during dissection. "M" denotes the mesometrial end, while "AM" denotes the anti-mesometrial end. Scale bars in stereoscope images are 400 μm.

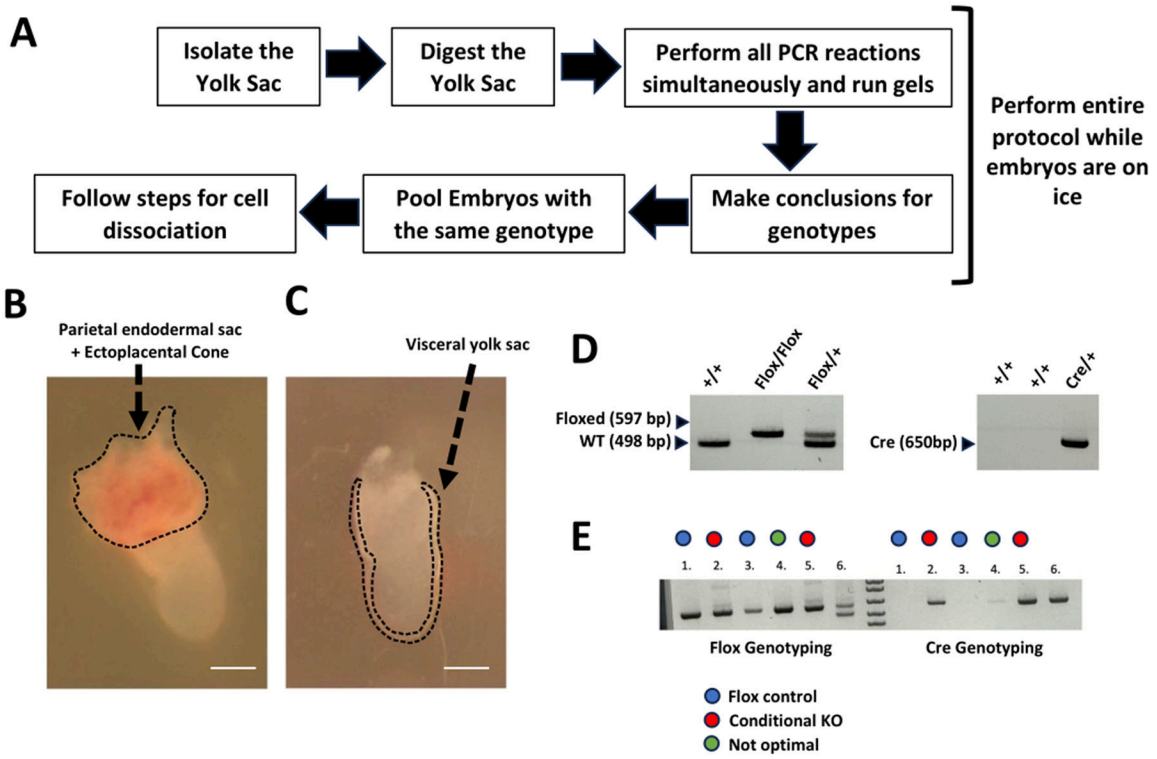

Figure 4: Same-day genotyping of yolk sacs from gastrulating mouse embryos.

(**A**) Workflow schematic for same-day genotyping of yolk sacs. (**B**) Representative image of the parietal endodermal sac and ectoplacental cone with associated maternal blood. (**C**) Visceral yolk sac in E7.5 embryos highlighted by the dashed line. Scale bar: 200 μm. (**D**) Representative gels of wild type (+/+), heterozygous (Flox/+), and homozygous flox (Flox/Flox), and Cre genotyping (Mesp1cre[15]) with observed DNA fragment sizes. (**E**) Same-day genotyping results for Cre and flox alleles from yolk sacs of 6 embryos (1-6) are provided. The two embryos with flox/flox and Cre negative (−) genotyping are flox controls (blue dots), while those with flox/flox and Cre positive (+) genotyping are conditional KOs (red dots). Embryo 4 is not optimal due to the faint Cre band (flox/flox and Cre-unclear) and is consequently excluded from further processing. Embryos with the same genotype are pooled and processed for single-cell RNA sequencing. Note that the presence of the Cre allele in the yolk sac does not affect the size of the flox bands , as the Cre used (Mesp1cre) is not expressed in the yolk sac.

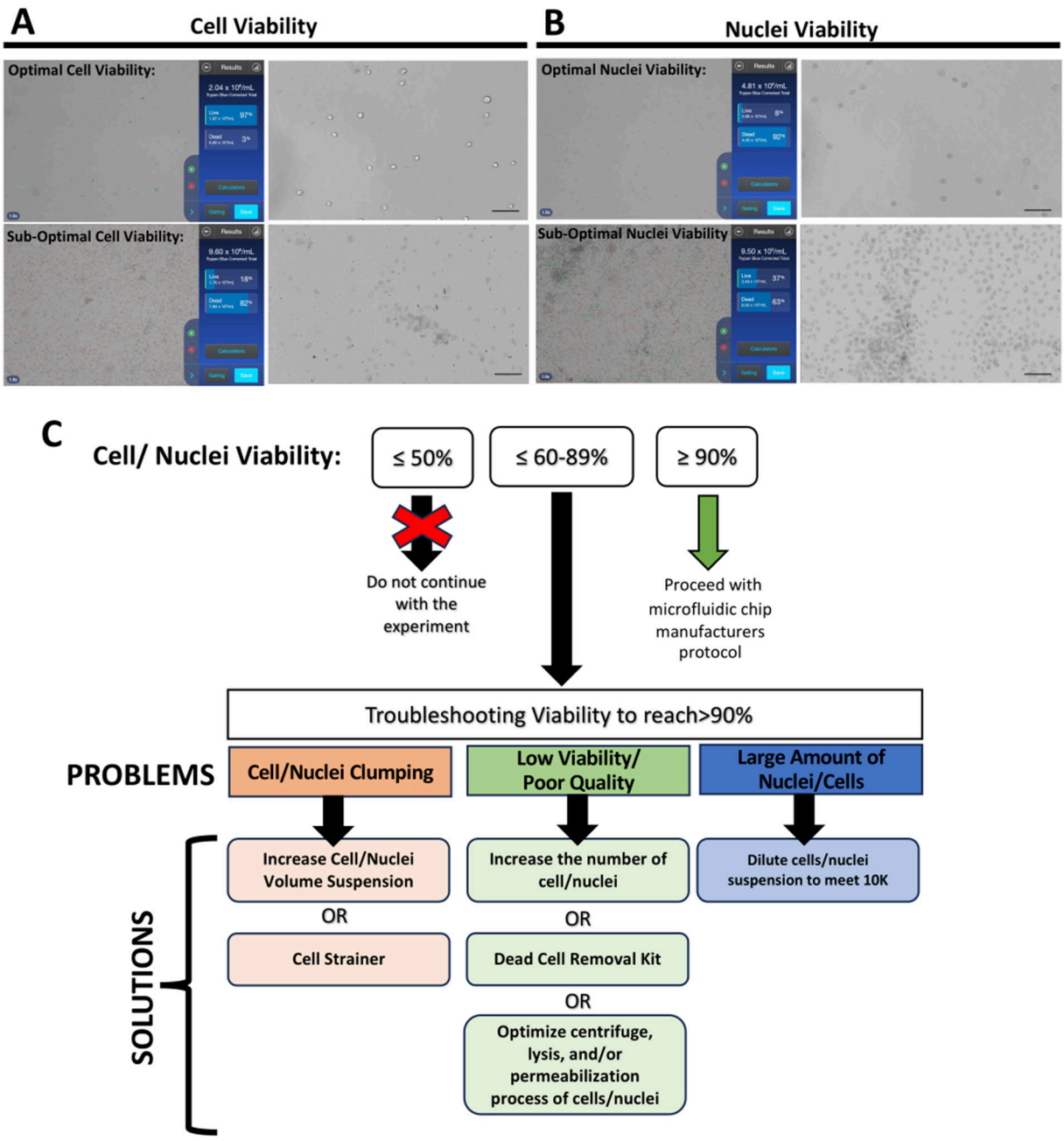

Figure 5: Assessment of cell quality and nuclei viability.

(**A**) Representative images of optimal and sub-optimal cell viability conditions from E7.5 embryos. (**B**) Representative images of optimal and sub-optimal nuclei viability conditions from E8 embryos (**C**) Troubleshooting scheme indicating potential solutions to help increase the viability of cells before starting single-cell partitioning.

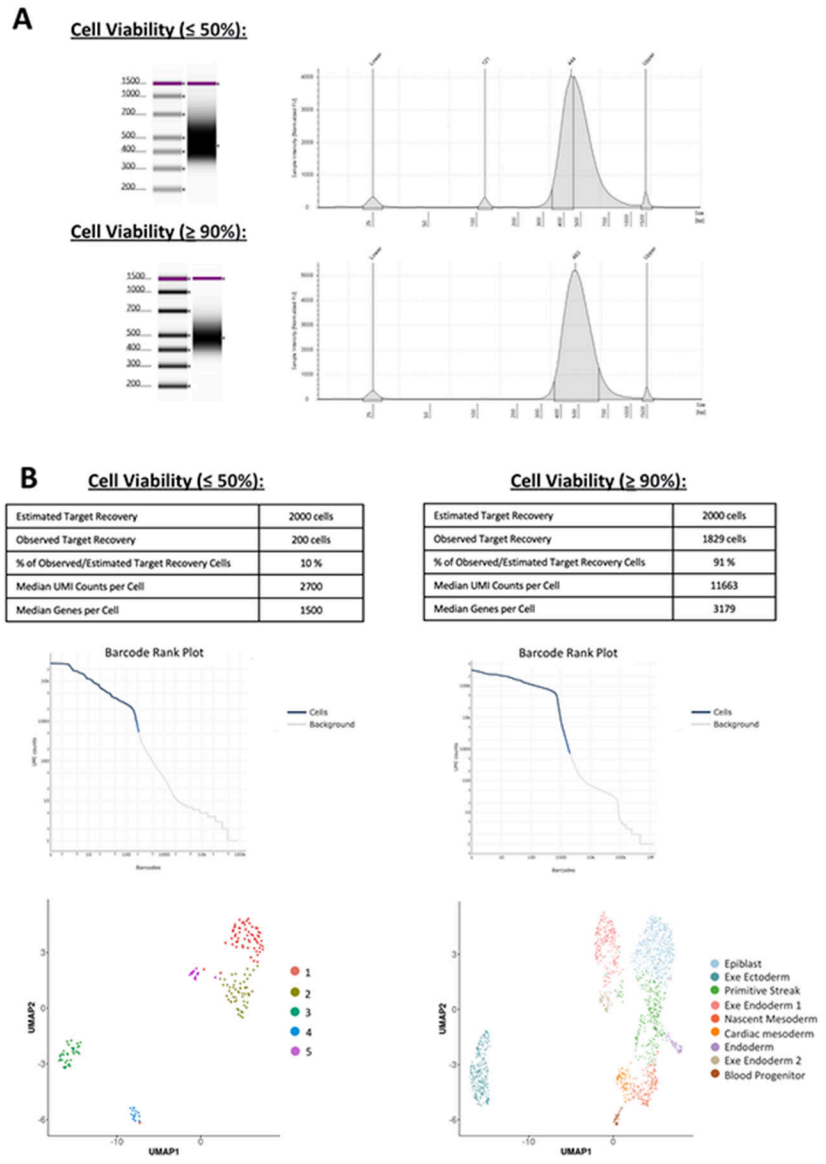

Figure 6: Single-cell RNA sequencing of E7 mouse embryos.

(**A**) Representative trace of fragment size distribution for single-cell RNA sequencing libraries from E7 mouse embryos for both sub-optimal and optimal conditions of cell viability, with the main peak near 400-500 bp. Note that the traces are similar between these conditions, indicating that the cell viability does not affect the quality controls of the library. (**B**) Analysis of single-cell RNA sequencing outcomes for E7 mouse embryos under both sub-optimal and optimal conditions showing observed target recovery, barcode ranking, and clustering of cell types through uniform manifold approximation and projection (UMAP) distribution.

Table 1:

Nuclei isolation lysis buffer and wash buffer composition.

Lysis Buffer			
Reagent Name	**Stock Concentration**	**Final Concentration**	**Total Volume 1 mL**
Tris-HCL (Ph7)	1 M	10 mM	10 µL
NaCl	5 M	10 mM	2 µL
MgCl2	0.1 M	3 mM	3 µL
Tween-20	20%	0.10%	5 µL
Nondiedt P40	10%	0.10%	10 µL
Digitonin	5%	0.01%	2 µL
DTT	1 M	1 mM	1 µL
RNase inhibitor	40 U/µL	1 U/µL	25 µL
Nuclease-free Water	--	--	924 µL
Wash Buffer			
Reagent Name	**Stock Concentration**	**Final Concentration**	**Total Volume 1 mL**
BSA in PBS	10%	1%	100 µL
PBS	--	--	900 µL